

Antiproliferation and Redifferentiation in Thyroid Cancer Cell Lines by Polyphenol Phytochemicals

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Thyroid carcinogenesis is accompanied by loss of thyroid-specific functions and refractory to radioiodine and thyroid stimulating hormone (TSH) suppression therapy. Redifferentiating agents have been shown to inhibit tumor growth and improve the response to conventional therapy. Polyphenol phytochemicals (PPs) in fruits and vegetables have been reported to inhibit cancer initiation, promotion, progression and induce redifferentiation in selected types. In this study we examined PPs induce redifferentiation in thyroid cancer cell lines. We investigated the effects of genistein, resveratrol, quercetin, kaempferol, and resorcinol on the F9 embryonal carcinoma cell differentiation model. The thyroid cancer cell lines, TPC-1, FTC-133, NPA, FRO, and ARO, displayed growth inhibition in response to genistein, resveratrol, quercetin. We further demonstrated that genistein decreased the dedifferentiation marker *CD97* in NPA cells and resveratrol decreased *CD97* in FTC-133, NPA, FRO cells and quercetin decreased *CD97* in all cell lines. We observed increased expression of differentiation marker *NIS* in FTC-133 cells in response to genistein, and resveratrol but no change in NPA, FRO, ARO cells. Quercetin increased or induced *NIS* in FTC-133, NPA, FRO cells. These findings suggest that PPs may provide a useful therapeutic intervention in thyroid cancer redifferentiation therapy.

Key Words: Thyroid Neoplasms; F9 Embryonal Carcinoma Cell; Redifferentiation; Polyphenols; Phytochemical; *CD97*; *Sodium-Iodine Symporter*

INTRODUCTION

Differentiated thyroid cancers (DTC) maintain thyroid specific functions even with regional metastasis, respond to surgical treatment, radioiodine therapy and TSH suppression therapy. However, poorly differentiated (PDTC) or anaplastic thyroid cancers (ATC) do not respond to conventional therapy, leading to poor outcome (1). During the dedifferentiation process, the cancer loses thyroid-specific gene expression, including the ability to absorb radioiodine and to express the TSH receptor; as such radioiodine therapy or thyroid stimulating hormone (TSH) suppressive therapy is not effective in PDTC/ATC. Dedifferentiated cancers display more rapid growth properties and are refractory to chemotherapy, external beam radiation therapy, and show poor outcomes in comparison with differentiated cancers.

Redifferentiation therapy is defined as a treatment to inhibit tumor growth and regain thyroid-specific functions in PDTC and ATC as a mean of increasing the response to conventional therapy (2). The goals of such therapy are to regain the expression of differentiation markers, including the sodium iodine symporter (NIS), and to reduce levels of the dedifferentiation marker *CD97*. Plasma membrane expression of NIS in thyroid

follicular cells is necessary to absorb iodine into the cell and, as such thyroid cancers with reduced NIS expression display impaired iodine uptake (3). *CD97* is a dedifferentiation marker that is increased during cancer progression and metastasis, and is reduced by redifferentiating agent such as retinoid acid (RA) in partially dedifferentiated thyroid cancer cells (4).

Currently available redifferentiating agents include RA, histone deacetylase inhibitors (HDAC inhibitors), and peroxisome proliferator activated receptor gamma (PPAR γ) agonist. Their effectiveness in dedifferentiated cancers and patient tolerability have been variable (1, 2, 5-7). Thus, this is a reason why more effective and better tolerated agents are needed.

The polyphenol phytochemicals (PPs) such as quercetin, resveratrol, genistein, and kaempferol (8-11), are present in fruits and vegetables, and inhibit cancer initiation, promotion and progression in vivo and in vitro. Moreover these compounds have been reported to induce redifferentiation in specific cancer types (12-14).

The F9 mouse embryonal carcinoma cell model (F9 cells) is frequently used to evaluate cellular morphological and molecular differentiation changes following drug and hormone treatment. The time duration of the growth cycle is very short and no

self differentiation is evident; however, drugs including RA have been demonstrated to induce differentiation after 72 hr of treatment. The primitive endoderm-like structure is formed with the expression of laminin B1, collagen type IV, and tissue plasminogen activator (tPA), followed by growth inhibition (15).

In the present study, we tried to demonstrate that some PPs have the ability to induce antiproliferation and redifferentiation in thyroid cancer cell lines using the F9 embryonal carcinoma cell differentiation model.

MATERIALS AND METHODS

Cell lines and culture

F9 embryonal cancer cells were cultured in DMEM (Sigma, St Louis, MO, USA) with 20% fetal bovine serum (FBS). TPC-1 (papillary thyroid cancer cell line) and FTC-133 (follicular thyroid cancer cell line), cells were cultured in DMEM with 10% FBS. NPA (poorly differentiated papillary thyroid cancer cell line), FRO (undifferentiated/anaplastic thyroid cancer cell line), and ARO (undifferentiated/anaplastic thyroid cancer cell line) cells were cultured in RPMI1640 (Sigma) with 10% FBS.

Reagents

Polyphenol phytochemical (resveratrol, genistein, quercetin, kaempferol, resorcinol (Sigma, Fig. 1) were dissolved in dimethylsulfoxide (DMSO; Sigma) at appropriate concentrations prior to dilution in tissue-culture medium such that the final concentration of DMSO not exceed 0.1% (v/v). Each reagent was assessed for a concentrations-dependent effect in F9 and thyroid cancer cells. In F9 cells, 0.2 μ M ATRA (all-trans-retinoic acid; Sigma) was used as a positive control.

Cell proliferation and growth inhibition

Cell proliferation and growth inhibition was measured by colorimetric dimethyl-thiazol-diphenyltetrazolium bromide (MTT) proliferation assays.

RT-PCR and semi quantitative analysis

At 72 hr after the administration of reagents, RT-PCR was performed for laminin B1, collagen type IV, and tissue plasminogen activator (tPA) mRNA in F9 cell. To assess thyroid cancer redifferentiation, RT-PCR was performed for *NIS*, *CD97* mRNA; *36B4*

mRNA was assessed as a housekeeping gene. RNA extraction and complementary DNA preparation were performed by conventional methods. The primers for each gene were designed and PCRs performed according to the following conditions (annealing temperature/cycles). *36B4*: forward 5'-CAGCTCTGGAGAACTGCTG-3', reverse 5'-GTGTACTIONCAGTCTCCACAGA-3' (49°C/25 cycles); laminin B1: forward 5'-TGAATTCTCACTGCAGACATGGTGAAG-3', reverse 5'-ATGGATCCCTCACTTATGTCCTTAAGGA-3' (48°C/30 cycles); collagen type IV: forward 5'-ATGAATTCTCAGCGTCTGGCTTCTGCT-3', reverse: 5'-ATGGATCCGTTGCATCCTGGGATACCTG-3' (62°C/30 cycles); *tPA* forward 5'-TTGGGCAGAACATACAGGGTGGTC-3', reverse 5'-CTGGCATGCATCGTGGAGGTCTT-3' (62°C/30 cycles); *NIS* forward 5'-GCCCTCATCCTGAACCAAGTG-3', reverse 5'-TGATCCGGAGTGGTTCTG-3' (50°C/30 cycles); *CD97* forward 5'-TCCTGCCGCGAGCTCCAACC-3', reverse 5'-GGCAGCGGCAGCTGTATGAAC-3' (58°C/30 cycles). PCR was conducted for the indicated number of cycles with denaturation at 95°C for 60 sec, annealing at the temperatures indicated for 60 sec, and extension at 72°C for 20 sec. Each RT-PCR experiment from F9 cell and thyroid cancer cells was performed in triplicate.

In thyroid cancer cell experiments, the PCR results were analyzed by semi quantitative methods using the Total Lab TL100 program (Nonlinear Dynamics Ltd., Newcastle, UK) compensated by comparing the *36B4* density of each PCR.

Statistical analyses

Statistical analyses were performed using the SPSS software (ver. 12.0 for Windows SPSS, Chicago, IL, USA). All MTT assays were performed in triplicate and mean value were compared using Student's t-test and one-way ANOVA. A *P* value < 0.05 was considered to indicate statistical significance.

RESULTS

F9 cell proliferation assays

We initially assessed the effects of resorcinol, genistein, resveratrol, kaempferol and quercetin on F9 cell proliferation at concentrations of 1, 10, 50, and 100 μ M respectively. Fig. 2 shows that resorcinol did not inhibit growth at concentrations below 100 μ M, while genistein, resveratrol, kaempferol and quercetin all inhibited cellular growth in a dose dependent manner. Fol-

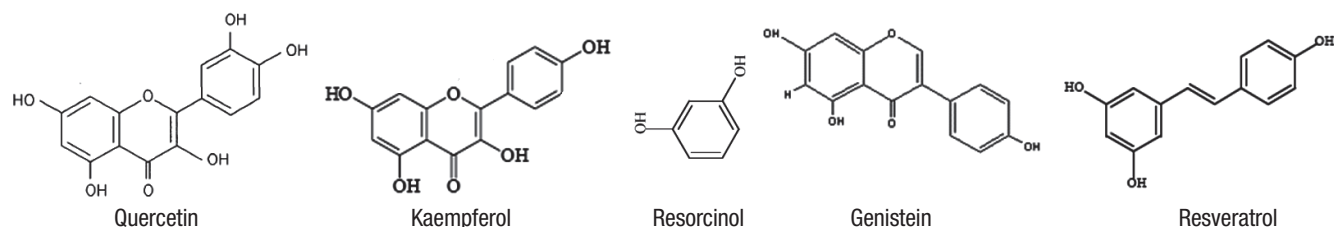


Fig. 1. Structure of polyphenol phytochemicals and resorcinol.

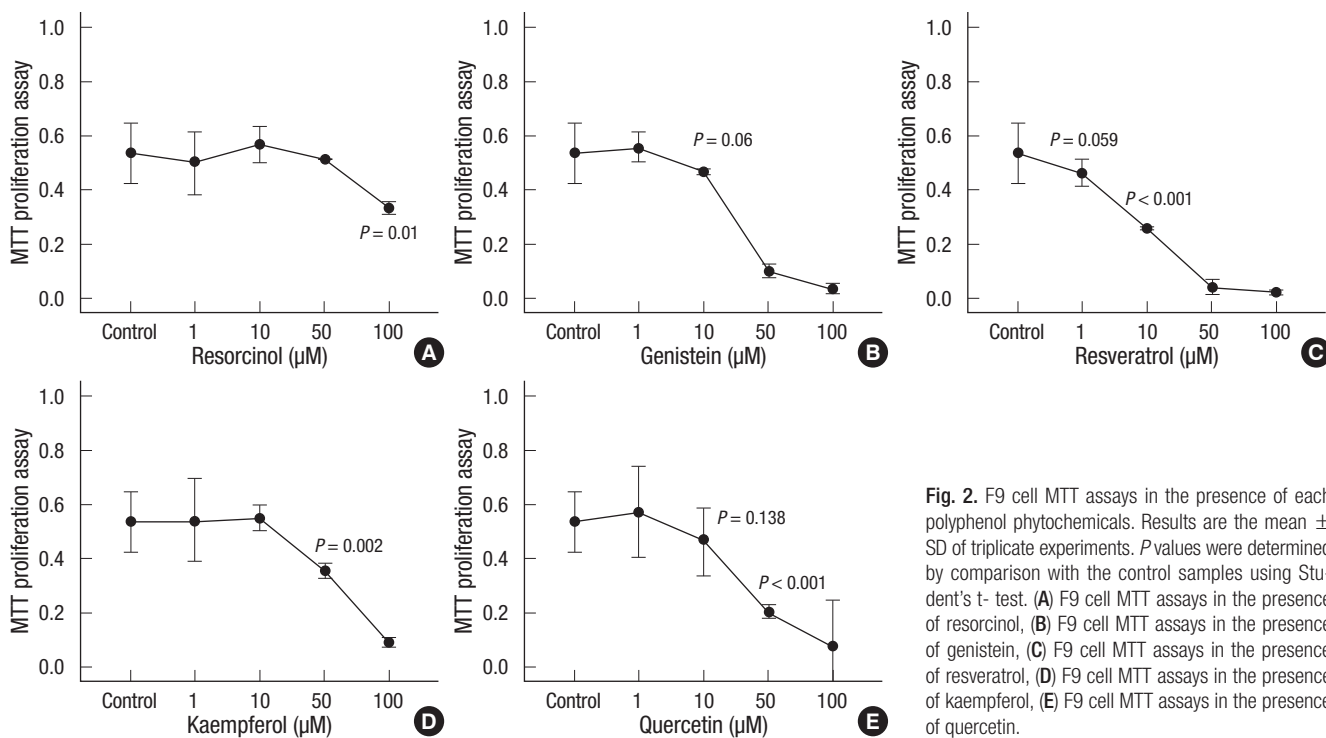


Fig. 2. F9 cell MTT assays in the presence of each polyphenol phytochemicals. Results are the mean \pm SD of triplicate experiments. *P* values were determined by comparison with the control samples using Student's *t*-test. (A) F9 cell MTT assays in the presence of resorcinol, (B) F9 cell MTT assays in the presence of genistein, (C) F9 cell MTT assays in the presence of resveratrol, (D) F9 cell MTT assays in the presence of kaempferol, (E) F9 cell MTT assays in the presence of quercetin.

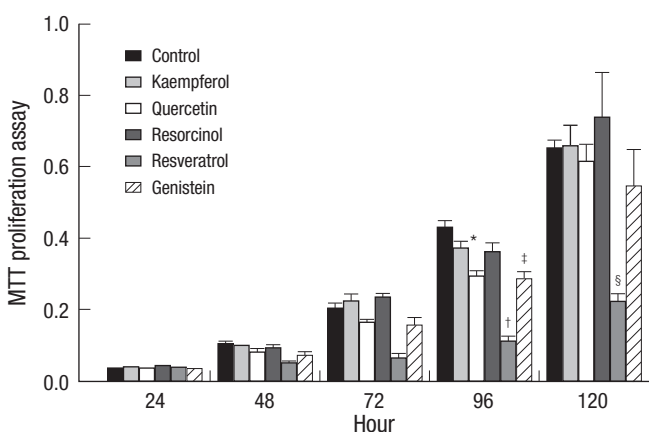


Fig. 3. F9 cell MTT assays in the presence of each polyphenol (10 μM). Results are the mean \pm SD of triplicate experiments. *P* values were determined by comparison with the control samples using Student's *t*-test. * $P = 0.001$; † $P < 0.001$; ‡ $P = 0.001$; § $P < 0.001$.

lowing the administration of each agent, MTT assays were performed at 24, 48, 72, 96, and 120 hr. Fig. 3 demonstrates that F9 cell growth was increased in a time-dependent manner in control cells (DMSO only), while quercetin, resveratrol and genistein inhibited cell growth up to 120 hr ($P < 0.05$). Kaempferol did not affect F9 cell growth.

We thus selected quercetin, resveratrol and genistein for further experiments.

Quantification of F9 cell differentiation markers by RT-PCR

Genistein, resveratrol and quercetin at concentration of 10 μM and ATRA (positive control) at a concentration of 0.2 μM were

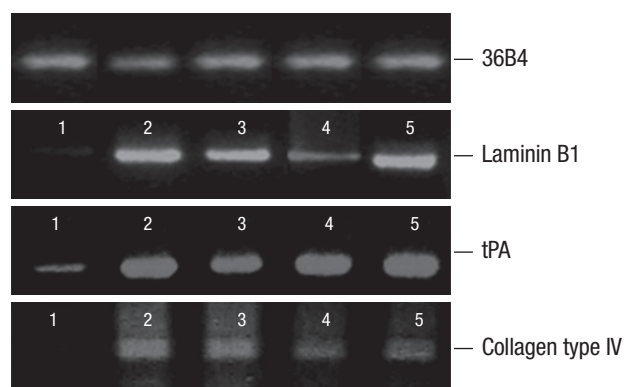


Fig. 4. F9 cell differentiation marker expression in the presence of ATRA and the polyphenol phytochemicals. Representative results are shown. 1, DMSO; 2, ATRA 0.2 μM; 3, Genistein 10 μM; Resveratrol 10 μM; Quercetin 10 μM.

administered to F9 cells in culture. After 72 hr, RT-PCR was performed for laminin B1, tPA and collagen type IV mRNA. Fig. 4 demonstrates that genistein, resveratrol and quercetin increased the levels of these differentiation markers compared with the DMSO-alone negative control.

Thyroid cancer cell proliferation assays

Fig. 5 demonstrates that when genistein and quercetin were administered for 72 hr, the growth of TPC-1, NPA, FTC-133, FRO, and ARO cells was inhibited in a dose-dependent manner ($P < 0.05$). Resveratrol inhibited the growth of these cells from 10 μM excluding TPC-1 cells that displayed increased levels of growth. TPC-1 proliferation was, however, inhibited at resveratrol concentrations of 50 μM or more.

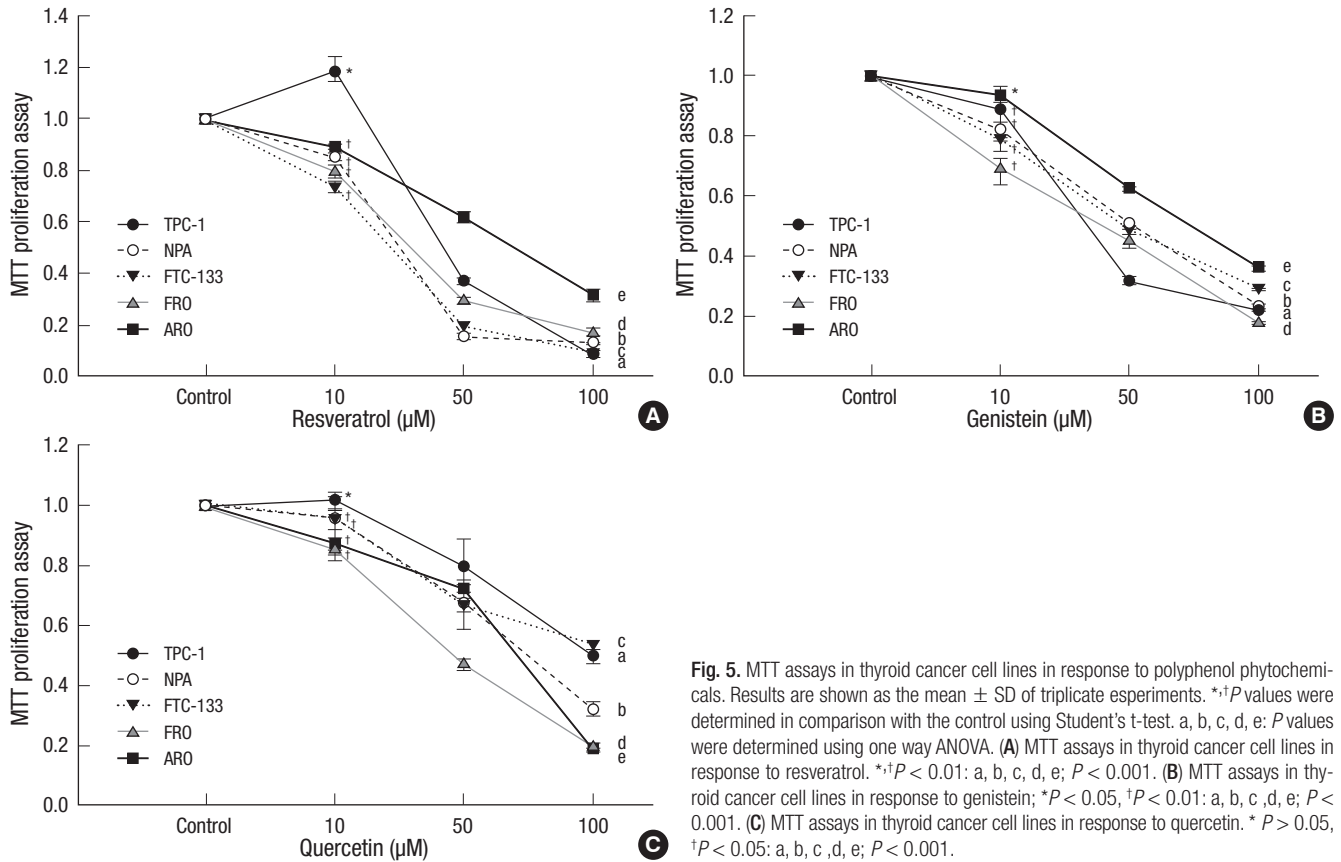


Fig. 5. MTT assays in thyroid cancer cell lines in response to polyphenol phytochemicals. Results are shown as the mean \pm SD of triplicate experiments. *[†]*P* values were determined in comparison with the control using Student's *t*-test. a, b, c, d, e: *P* values were determined using one way ANOVA. (A) MTT assays in thyroid cancer cell lines in response to resveratrol. *[†]*P* < 0.01; a, b, c, d, e; *P* < 0.001. (B) MTT assays in thyroid cancer cell lines in response to genistein; **P* < 0.05, [†]*P* < 0.01; a, b, c, d, e; *P* < 0.001. (C) MTT assays in thyroid cancer cell lines in response to quercetin. * *P* > 0.05, [†]*P* < 0.05; a, b, c, d, e; *P* < 0.001.

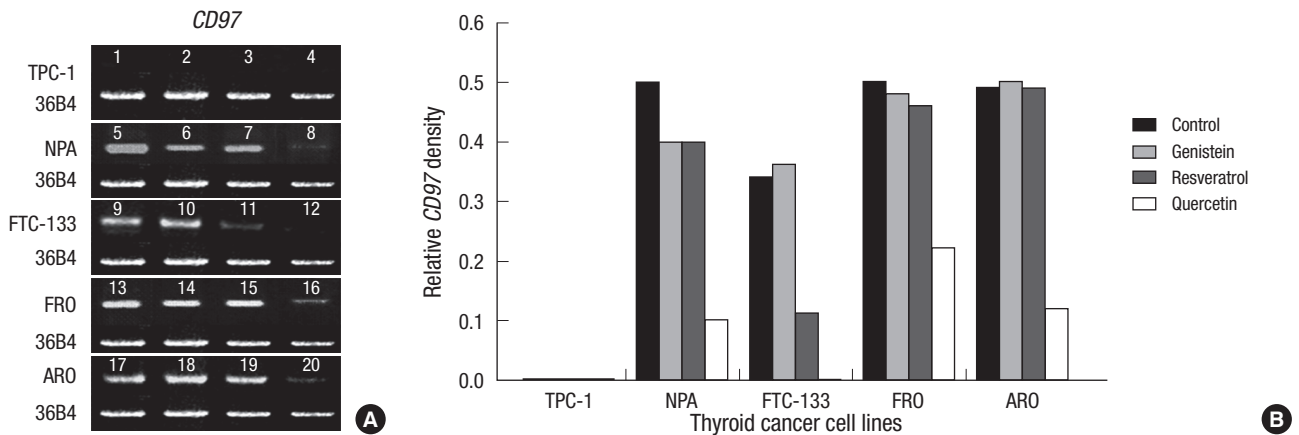


Fig. 6. RT-PCR for *CD97* mRNA in thyroid cancer cell lines. (A) RT-PCR for *CD97* mRNA levels in thyroid cancer cell lines following genistein, resveratrol or quercetin treatment (1, Control; 2, Genistein 100 µM; 3, Resveratrol 50 µM; 4, Quercetin 100 µM; 5, Control; 6, Genistein 20 µM; 7, Resveratrol 50 µM; 8, Quercetin 100 µM; 9, Control; 10, Genistein 20 µM; 11, Resveratrol 20 µM; 12, Quercetin 20 µM; 13, Control; 14, Genistein 50 µM; 15, Resveratrol 50 µM; 16, Quercetin 20 µM; 17, Control; 18, Genistein 50 µM; 19, Resveratrol 20 µM; 20, Quercetin 20 µM). (B) Relative *CD97* density in thyroid cancer cell lines following genistein, resveratrol or quercetin treatment. Results are normalized to the relative density of *36B4*.

Assessment the expression of thyroid cancer differentiation markers by RT-PCR

RT-PCR for *CD97* and *NIS* mRNA was performed following 72 hr of treatment for TPC-1 cells: genistein (100 µM), resveratrol (50 µM), quercetin (100 µM), for NPA cells: genistein (20 µM), resveratrol (50 µM), quercetin (100 µM), for FTC-133 cells: genistein (20 µM), resveratrol (20 µM), quercetin (20 µM), for FRO cells: genistein (50 µM), resveratrol (50 µM), quercetin (20 µM),

for ARO cells: genistein (50 µM), resveratrol (20 µM), quercetin (20 µM). In contrast to the other cell lines under investigation, TPC-1 cells did not express the dedifferentiation marker *CD97*. We observed a decrease in *CD97* expression following genistein treatment in NPA cells. Resveratrol decreased *CD97* in FTC-133, NPA and marginally decreased *CD97* in FRO cells, while quercetin decreased *CD97* levels in FTC-133, NPA, FRO, and ARO cells. Expression of *CD97* in TPC-1 cells was unaffected by the pres-

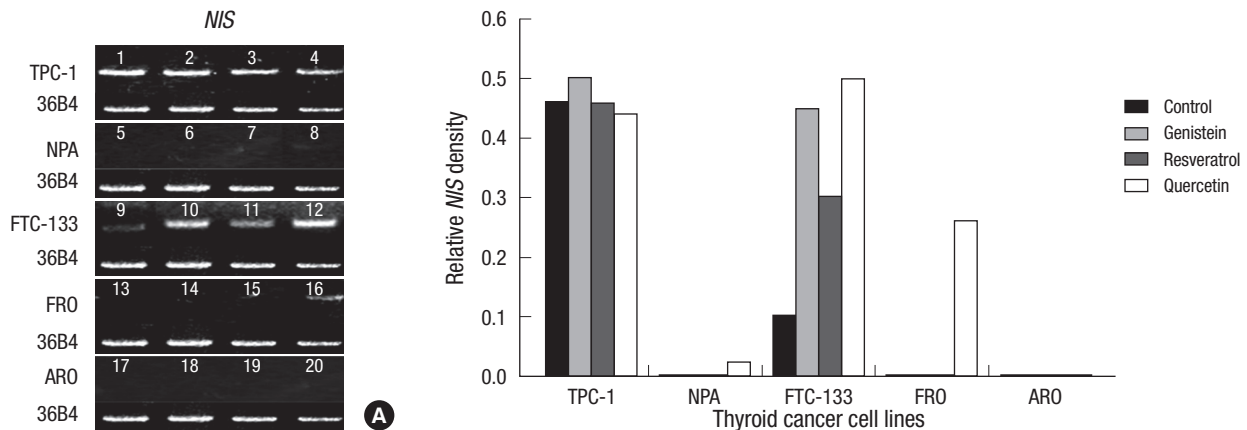


Fig. 7. RT-PCR for *NIS* mRNA in thyroid cancer cell lines. (A) RT-PCR for *NIS* mRNA in thyroid cancer cell lines following genistein, resveratrol or quercetin treatment (1, Control; 2, Genistein 100 μ M; 3, Resveratrol 50 μ M; 4, Quercetin 100 μ M; 5, Control; 6, Genistein 20 μ M; 7, Resveratrol 50 μ M; 8, Quercetin 100 μ M; 9, Control; 10, Genistein 20 μ M; 11, Resveratrol 20 μ M; 12, Quercetin 20 μ M; 13, Control; 14, Genistein 50 μ M; 15, Resveratrol 50 μ M; 16, Quercetin 20 μ M; 17, Control; 18, Genistein 50 μ M; 19, Resveratrol 20 μ M; 20, Quercetin 20 μ M). (B) Relative *NIS* density in thyroid cancer cell lines following genistein, resveratrol or quercetin treatment. Results are normalized to the relative density of *36B4*.

ence of any of the PPs (Fig. 6). *NIS* was expressed in TPC-1 and FTC-133 cells. While the levels of *NIS* were unchanged in the presence of genistein, resveratrol and quercetin in TPC-1 cells, all three compounds increased *NIS* levels in FTC-133 cells. Genistein and resveratrol both failed to induce in NPA, FRO or ARO cells. Quercetin did not induce *NIS* in ARO cells but did induce *NIS* in dedifferentiated/anaplastic NPA and FRO cells (Fig. 7).

DISCUSSION

RA, HDAC inhibitor, and PPAR γ agonists are effective redifferentiation therapies in partially dedifferentiated thyroid cancers, but are ineffective undifferentiated/anaplastic cancers and display high patient toxicity or effectiveness only in vitro. Polyphenol phytochemicals have been reported to inhibit cancer initiation, promotion and progression in vivo or in vitro and moreover have been reported to induce redifferentiation in specific cancer types (12-14). We present the first report that identifies the ability of PPs to promote tumor growth inhibition in both partially dedifferentiated cancer and dedifferentiated/anaplastic cancers.

We demonstrated that genistein (rich in soybeans), resveratrol (rich in grapes), quercetin (rich in onions) inhibit the tumor growth of TPC-1, FTC-133, NPA, FRO, and ARO cell lines. The mechanism of the growth inhibition of genistein, resveratrol and quercetin is thought to involve the inhibition of epidermal growth factor (EGF), NF- κ B, cyclin D and protein tyrosine kinase, all of which increase tumor promotion, progression, and increases caspase which can induce apoptosis (16-18). They have been tested in normal and cancer cultured cells, and shown that these compounds induced apoptosis in cultured cancer cells but not in their normal counterparts up to 100 μ M, so these effects were not by general toxicity (19). Other studies have suggested that

PPs act as phytoestrogens and inhibit the estrogen-dependent growth of estrogen receptor (ER) - positive cancer cells (20) and specific cancer cells (NPA, FRO) that express ER (11). We observed increased TPC-1 cell growth in response to low dose resveratrol (10 μ M), most likely caused by its phytoestrogenic effects, stimulating the estrogen receptor α (ER α) and inhibitory estrogen receptor β (ER β). The dose of resveratrol displays biphasic effects in MCF-7 breast cancer cells and low dose are thought to affect growth stimulation (21). However, no report has described the expression of ER in TPC-1 cells.

CD97 is a representative dedifferentiation marker in thyroid cancers that is stimulated by EGF, and can be inhibited genistein, resveratrol and quercetin (16), consistent with their effects on cancer cell observed in our study.

The dedifferentiation process is accompanied by both genetic mutations and epigenetic changes. During dedifferentiation process, a loss of *NIS* gene expression occurs, caused by hypermethylation of the *NIS* gene; this is recovered by demethylating agent 5-azacytidine (22). Additionally, *NIS* expression is induced by TSH mediated by TSH receptor (TSHR). Dedifferentiated thyroid cancers are accompanied by a loss of the *TSHR* gene promoter hypermethylation and is restored by 5-azacytidine (23). DNA methylation is mediated by DNA methyltransferases (DNMT) and numerous cancers including thyroid cancers, have been reported to have stimulated hypermethylation (22, 23). In this study, genistein, resveratrol and quercetin all increased *NIS* expression in FTC-133 cells while quercetin also increased *NIS* expression in dedifferentiated/anaplastic NPA and FRO cells. Quercetin has been reported to reverse DNA hypermethylation in a dose-dependent manner in other cancers (24). Genistein has been reported to reverse retinoic acid receptor β (*RAR* β) promoter hypermethylation and increase *RAR* β in prostate and esophageal cancers (25). There is no report regarding the effects

of resveratrol in relation to DNA methylation; however, most of PPs are substrates of catechol *O*-methyltransferase (COMT) and are converted to *S*-adenosyl-*L*-homocysteine (SAH) by COMT, an antagonist of DNMT (24). Additionally, resveratrol has been reported to increase the transcription of *PPAR* γ , which can induce thyroid cancer redifferentiation (26). RA acts through binding with *RAR* β and has been reported to restore *NIS* expression in FTC-133 (27), but not in NPA, FRO or ARO cells. Additionally RA does not inhibit growth of FRO and ARO cells that have lost *RAR* β (28), which occurs as a result of promoter hypermethylation in numerous thyroid cancer cell lines (29). Thus, PPs that inhibit *RAR* β hypermethylation, when administered in combination with low-dose RA, may be effective in thyroid cancers that are refractory to RA therapy. Serum concentrations of PPs typically do not exceed 10 μ M in humans, a concentration that is both antiproliferative and induces redifferentiation *in vitro*. The tissue concentration levels of these compounds can increase 1-10 fold following dietary supplementation (30).

In conclusion these findings suggest that genistein, resveratrol and quercetin may be effective for thyroid cancer redifferentiation therapy, including of undifferentiated/anaplastic cancers. Further investigations are required to increase the tissue concentrations of these polyphenol phytochemicals in thyroid cancer patients and to assess their effectiveness in patients with dedifferentiated thyroid cancers.

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AUTHOR SUMMARY

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This study assessed the ability of polyphenol phytochemicals (PPs) as a redifferentiation therapy in thyroid cancer cells. Using F9 cell differentiation model for the selection of effective PPs, we demonstrated that genistein, resveratrol, quercetin were effective. In five kinds of thyroid cancer cell lines, we further demonstrated that the PPs not only decrease the dedifferentiation markers, but also increase the expression of differentiation marker. These findings suggest that the PPs investigated in this study might be considered for the redifferentiation therapy of thyroid cancer.